Sevoflurane reduces severity of acute lung injury possibly by impairing formation of alveolar oedema

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Abstract: Pulmonary oedema is a hallmark of acute lung injury (ALI), consisting of various degrees of water and proteins. Physiologically, sodium enters through apical sodium channels (ENaC) and is extruded basolaterally by a sodium-potassium-adenosine-triphosphatase pump (Na(+) /K(+) -ATPase). Water follows to maintain iso-osmolar conditions and to keep alveoli dry. We postulated that the volatile anaesthetic sevoflurane would impact oedema resolution positively in an in-vitro and in-vivo model of ALI. Alveolar epithelial type II cells (AECII) and mixed alveolar epithelial cells (mAEC) were stimulated with 20 µg/ml lipopolysaccharide (LPS) and co-exposed to sevoflurane for 8 h. In-vitro active sodium transport via ENaC and Na(+) /K(+) -ATPase was determined, assessing (22) sodium and (86) rubidium influx, respectively. Intratracheally applied LPS (150 µg) was used for the ALI in rats under sevoflurane or propofol anaesthesia (8 h). Oxygenation index (PaO(2) /FiO(2)) was calculated and lung oedema assessed determining lung wet/dry ratio. In AECII LPS decreased activity of ENaC and Na(+) /K(+) -ATPase by $17 \cdot 4\% \pm 13 \cdot 3\%$ standard deviation and $16 \cdot 2\% \pm 13 \cdot 1\%$, respectively. These effects were reversible in the presence of sevoflurane. Significant better oxygenation was observed with an increase of PaO(2) /FiO(2) from 189 ± 142 mmHg to 454 ± 25 mmHg after 8 h in the sevoflurane/LPS compared to the propofol/LPS group. The wet/dry ratio in sevoflurane/LPS was reduced by $21 \cdot 6\% \pm 2 \cdot 3\%$ in comparison to propofol/LPS-treated animals. Sevoflurane has a stimulating effect on ENaC and Na(+) /K(+) -ATPase in vitro in LPS-injured AECII. In-vivo experiments, however, give strong evidence that sevoflurane does not affect water reabsorption and oedema resolution, but possibly oedema formation.

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Sevoflurane reduces severity of acute lung injury possibly by impairing formation of alveolar edema

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Abstract

Pulmonary edema is a hallmark of acute lung injury (ALI), consisting of various degrees of water and proteins. Physiologically, sodium enters through apical sodium channels (ENaC) and is extruded basolaterally by a sodium-potassium-adenosine-triphosphatase pump (Na^+/K^+-ATPase). Water follows to maintain isoosmolar conditions and to keep alveoli dry. We postulated that the volatile anesthetic sevoflurane would positively impact edema resolution in an *in vitro* and *in vivo* model of ALI.

Alveolar epithelial type II cells (AECII) and mixed alveolar epithelial cells (mAEC) were stimulated with 20 µg/ml lipopolysaccharides (LPS) and co-exposed to sevoflurane for 8 hours. *In vitro* active sodium transport via ENaC and Na^+/K^+-ATPase was determined, assessing ^{22}sodium and ^{86}rubidium influx, respectively. Intratracheally applied LPS (150 µg) was used for the *in vivo* ALI in rats under sevoflurane or propofol anesthesia (8 hours). Oxygenation index (PaO_2/FiO_2) was calculated and lung edema was assessed determining lung wet/dry ratio.

In AECII LPS decreased activity of ENaC and Na^+/K^+-ATPase, both by 17% and 16%, respectively ($p<0.05$). These effects were reversible in the presence of sevoflurane ($p<0.05$). Considerably better oxygenation was observed with an increase of PaO_2/FiO_2 from 189 mmHg to 454 mmHg after 8 hours ($p<0.05$) in the sevoflurane/LPS compared to propofol/LPS group. Wet/dry-ratio in sevoflurane/LPS was reduced by 21.6% in comparison to propofol/LPS-treated animals ($p<0.05$).

Sevoflurane has a stimulating effect on ENaC and Na^+/K^+-ATPase *in vitro* in LPS-injured AECII. *In vivo* experiments, however, give strong evidence that sevoflurane does not impact on water reabsorption and edema resolution, but possibly on edema formation.

Introduction

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are a major cause of acute respiratory failure in critically ill patients [1]. Mortality of ARDS remains high since its first description by Ashbaugh and colleagues [2], although lung protective ventilatory strategies have reduced mortality from 60-70% to 35-40% [3], [4]. While drug treatment is intensively investigated, no pharmacological approach has been established yet [5-8].

ALI/ARDS is characterized by capillary leak and reduced fluid reabsorption, resulting in lung edema. The level of decreased rate of fluid clearance has significant prognostic value for morbidity and mortality [9]. In addition to reduced fluid reabsorption protein clearance is impaired as well. As shown in patients with ARDS non-survivors have three times higher alveolar protein concentrations than survivors [10,11].

Several studies have tried to detect the underlying mechanism of impairment of alveolar fluid clearance in ALI/ARDS and various pathways have been suggested [12-14]. According to experimental evidence, the active sodium (Na^+) transport is thereby the most important ion transport mechanism involved in fluid reabsorption out of the alveolar space [15,16]. The broadly accepted paradigm for Na^+ transport in the alveoli is a two-step process: Na^+ enters the cell by epithelial amiloride-sensitive Na^+-channels (ENaC) located at the apical surface and is extruded by basolaterally located sodium-potassium-adenosine-triphosphatase pumps (Na^+/K^+-ATPases) [17,18]. Research done in the last two decades has shown that this vectorial transport from apical to basal generates the osmotic force for water flow out of the alveolar air spaces [19-22]. This process can be up- or down regulated, implying an increased or diminished clearance of alveolar fluid. Studies have demonstrated that net vectorial fluid transport is reduced in human alveolar epithelial cells type II (AEC II) in ALI [23].

Patients suffering from ALI/ARDS most often need to be mechanically ventilated and therefore remain sedated in intensive care units (ICU) [24]. The overall effect of sedatives and anesthetics - volatile anesthetics included - on this disease is unclear. As previously demonstrated the inflammatory response upon endotoxin stimulation in AEC is partly

reversible in the presence of sevoflurane [25]. In an *in vivo* model of ALI oxygenation improved in the presence of sevoflurane [26]. However, at the same time, volatile anesthetics are suspected to impair sodium transport [27].

Aim of this work was to investigate the effect of the nowadays commonly used volatile anesthetic sevoflurane on ENaC and Na^+/K^+-ATPase *in vitro* and *in vivo*. Based on our previous *in vitro* and *in vivo* results with a positive effect of sevoflurane [26] we hypothesized that *in vitro* activity of ENaC and Na^+/K^+-ATPase in endotoxin-injured AEC may be increased upon treatment with sevoflurane. Furthermore, we tried to clarify the impact of sevoflurane on edema *in vivo* in the endotoxin-induced lung injury model. We postulated that sevoflurane would improve alveolar fluid clearance.

Materials and methods

1 In vitro assay

Alveolar epithelial cells type II (AECII)

The L2 cell line (CCL 149; American Type Culture Collection, Rockville, MD, USA) was derived trough cloning of adult female rat lung of AEC type II origin. Cells were cultured in Dulbecco's modified Eagle's Medium (DMEM, Invitrogen Corporation, Carlsbad, CA, USA), supplemented with 10% foetal bovine serum (FBS, Invitrogen Corporation, Carlsbad, CA, USA), 1% penicillin-streptomycin and 1% 4-(2-hydroxethyl)-1-piperazineethanesulfonic acid buffer (HEPES, Invitrogen Corporation, Carlsbad, CA, USA). They were grown for 3 days in uncoated plates (Corning Inc., Croning, NY, USA) to > 95% confluence.

Mixed alveolar epithelial cells (mAEC)

Primary AEC were harvested following an established protocol [28, 29]. Briefly, lungs were explanted from male Wistar rats, injected with 10 ml of phosphate-buffered saline (PBS) containing 4U/ml porcine pancreas elastase (Sigma-Aldrich, Hamburg, Germany), and incubated for 20 minutes at 37°C. Trachea and large airways were discarded and lungs were minced. Elastase reaction was stopped with 5 ml FBS. After vigorous stirring for 20 minutes, cells were filtered and incubated for 1 hour at 37°C in petri dishes, previously coated with 1 mg/ml rat IgG (Sigma-Aldrich, Hamburg, Germany) in PBS, in order to remove immunocompetent cells. Unattached cells were washed away, and the remaining cells were cultured in DMEM/10%FBS. After an incubation time of 7 days a mixture of type I and type II cells (mAEC) were found (**Figure 1**). Type II character was detected analyzing sodium-dependent phosphate-cotransporter type IIb (NaPi IIb) [30,31], type I cells with detection of aquaporin 5 (AQP5) [32,33].

Stimulation with LPS and sevoflurane exposure

DMEM/10% FBS of confluent AEC-monolayers was replaced by DMEM/1% FBS at least 14 hours before starting the experiment. AEC were stimulated with lipopolysaccharide (LPS) from *Escherichia coli,* serotype 055:B5 (Sigma-Aldrich, Hamburg, Germany) in a concentration of 20 µg/ml in DMEM/1% FBS (control group with PBS) according to previous publications [34,35], and placed in two humidified, preheated (37°C) air-tight chambers (oxid anaerobic jar; Oxoid AG, Basel, Switzerland).

AEC were exposed to 1 minimal alveolar concentration (MAC) = 2.2 vol. % sevoflurane (Sevorane®, Abbott AG, Baar, Switzerland) for 8 hours, representing a clinically relevant concentration of the volatile anesthetic as used in previously performed experiments [34]. A mixture of 5% CO_2 and 95% air was directed trough a Sevotec 5 Vaporizer (Abbott, Switzerland), placed at the entrance of the chamber (for control only CO_2 /air mixture). Within 5 min, sevoflurane reached the steady state concentration of 2.2 vol. % (monitored by Ohmedia 5330 Agent Monitor; Abbott AG). The chambers were sealed for 8 hours and incubated at 37°C. At the end of the experiment sevoflurane concentration was verified again to confirm the value of 2.2 vol%.

^{22}Na influx studies

Measurement of ^{22}Na flux trough amiloride-sensitive Na^+ channels was performed as described [36]. Culture medium was removed, and cells on 6-well plates were rinsed twice and preincubated at 37°C for 20 min in a buffered sodium-free solution containing (in mM): 137 N-methylglucamine, 5.4 KCl, 1.2 $MgSO_4$, 2.8 $CaCl_2$ and 15 Hepes (pH 7.4). This solution was replaced by uptake solution composed of (in mM): 14 NaCl, 35 KCl, 96 N-methylglucamine and 20 HEPES (pH 7.4) containing 0.5 µCi/ml of ^{22}NaCl (37 MBq/mg Na) in absence or presence of 100 µM amiloride. Amiloride blocks sodium uptake via ENaC and was used as positive control for blocking sodium absorption. After an incubation time of 5 min cells were washed twice with 1 ml/well of an ice-cold solution containing (in mM): 120 N-methylglucamine and 20 HEPES (pH 7.4). Cells were

solubilized in 0.3 ml/well trypsin for 3 min. Tracer activities were determined by liquid scintillation counting (Tri-carb 2900TR, liquid scintillation scanner, Packard, Chicago, IL, USA).

^{86}Rubidium influx studies

The measurement of ouabain-sensitive rubidium (^{86}Rb) influx was performed as previously described [37,38]. Assays were performed in a buffered solution A of the following composition (in mM): 120 NaCl, 5 RbCl, 1 $MgSO_4$, 0.15 Na_2HPO_4, 0.2 NaH_2PO_4, 4 $NaHCO_3$, 1 $CaCl_2$, 5 glucose, 2 lactate, 4 essential and nonessential amino acids, 20 HEPES, and 0.1% bovine serum albumin (BSA). The osmotic pressure of solution A was adjusted by mannitol to 350 mosM, pH was 7.4. After removal of the culture medium half of the cells in 6-well plates were incubated with 1 ml/well of 4 mM ouabain at 37°C for 30 minutes. Ouabain blocks Na^+/K^+-ATPase and was used as positive control for blocking the transporter. The other half was incubated with solution A. Subsequent plates were washed with 1 ml/well of solution A and incubated 5 min with 0.6 ml/well of Solution A supplemented with 1 µCi/ml ^{86}RbCl (370 MBq/mg Rb). Uptake was stopped by washing the cells twice with 1 ml/well of ice-cold rinsing solution containing the following (in mM): 140 N-methylglucamine, 1.2 $MgCl_2$, 3 $NaCl_2$, 10 Hepes, and 0.1% BSA at pH 7.4. Solubilized cells were traced by liquid scintillation counting.

All chemicals were purchased from Sigma-Aldrich, Hamburg, Germany, culture media and their reagents from Invitrogen Corporation, Carlsbad, CA, USA. Radioactive tracers were supplied by PerkinElmer AG, Schwerzenbach, Switzerland.

Statistical analysis

Each experimental setup was performed three times, each conducted in sextuplets. Data of the three experiments were taken together and analysed (n=18). Values are expressed as mean +/- SD. Optical analysis of boxplots suggested normal distribution of data. Confirmation was done by a Shapiro Wilk Test. The effects of sevoflurane were compared

with the control group (PBS group) for K^+ - and Na^+ -influx and tested by analysis of variances for repeated measurements (one-way ANOVA), including a Tukey-Kramer Multiple comparison Test. Graphpad Prism4® Graphpad Instat3® (GraphPad software, La Jolla, CA, USA) was used for statistical analyses. P-values <0.05 are considered statistically significant.

2 In vivo assay

Animal preparation

After approval from the local animal care and use committee (Zürich, Switzerland) experiments were performed with pathogen-free, male Wistar rats (Charles River, Sulzfeld, Germany), (body weight 350 -500 g). The rats were kept in standard cages at 22 °C (12 hours light /12 hour dark). Food and water were supplied *ad libitum*.

Induction of anesthesia and monitoring was performed as previously described [26]. Rats were tracheotomized. After insertion of a sterile metal cannula, animals were ventilated in parallel (Servo Ventilator 300, Maquet, Solna, Sweden). Pressure controlled ventilation was set with 30 breathes per minute, pressure was 3/14 cm H_2O, inspiration to expiration-ratio 1:2, and fractional inspired oxygen concentration (FiO_2) was 100%. Arterial blood was analysed at 0, 2, 4, 6 and 8 hours. Using 100% FiO_2 during the whole experiment, the oxygen capability of the lung is represented by the oxygen tension (PaO_2 in mmHg) in arterial blood gas samples (oxygenation index: PaO_2/FiO_2). Body temperature was controlled by rectal temperature measurement and corrected to 37°C by a heating lamp.

Experimental design

Rats were randomized into three different groups, using sealed envelopes: a) propofol/PBS; b) propofol/LPS and c) sevoflurane/LPS (n=6 in all groups). Rats were intratracheally instilled with 150 µg LPS in 300 µl PBS (control with PBS only) [39], immediately followed by randomisation in either propofol or sevoflurane group (co-conditioning). Anesthesia was performed as described [26].

RNA extraction and real-time PCR for α-ENaC, γ-ENaC and α_1-Na^+/K^+-ATPase

Eight hours after the onset of injury rats were euthanized and lungs were explanted, shock-frozen in liquid nitrogen and stored at -80°C for isolation of mRNA.

Total RNA was isolated form lung tissue using the RNeasy® Mini Kit (Qiagen, Basel, Switzerland) according to the manufacture's protocol. RNA amounts were determined by absorbance at 260 nm.

Reverse transcription and real-time quantitative TaqMan™ polymerase chain reaction (PCR) were performed as described [26]. Specific primers (Microsynth, Balgach, Switzerland) and labeled TaqMan probes (Roche Applied Science, Switzerland) were designed for α- and γ- subunits of ENaC, for α_1-subunit of Na^+/K^+-ATPase and 18S as housekeeping gene. All primers and probes used in the experiments are presented in **Tab. 1**. Each experimental PCR run was performed in duplicate with simultaneous negative controls without template.

For quantitation of gene expression the comparative C_t method was used as described by Livak et al. [40]. The C_t values of samples (propofol/LPS and sevoflurane/LPS) and control (propofol/PBS) were normalized to the housekeeping gene (18S) and calculated as follows: $2^{-[delta]\,[delta]\,Ct}$, where $[delta][delta]C_t = [delta]C_{t,\,samples} - [delta]C_{t,\,controls.}$

Lung wet/dry ratio

Sevoflurane/LPS animals were given 150 µg LPS in 300 µl PBS with or without 100 µM amiloride to block sodium resorption via ENaC [41] (Sigma-Aldrich, Hamburg, Germany). After 8 hours animals were sacrificed, lungs were explanted, and wet weight was measured. Thereafter, lungs were air dried for 72 hours at 65°C and lung dry weight was quantified. Wet/dry ratio (w/d) was calculated as follows [42]: $w/d = weight_{wet} / weight_{dry}$

Statistics

Values are expressed as mean ± SD, n=6 per group. Optical analysis of boxplots suggested normal distribution of data. Confirmation was done by a Shapiro Wilk test. Vital parameters were tested by analysis of variances for repeated measurements (one-way ANOVA) with a Tukey-Kramer Multiple post-hoc test. Real-time PCR and wet/dry-ratio data were tested using student's T-test. Graphpad Prism4® and Graphpad Instat3® (GraphPad software, La Jolla, CA, USA) were used for statistical analyses. P-values less or equal to 0.05 are considered statistically significant.

Results

Cell survival

As described in previous experiments [34,25], cell survival was not influenced upon sevoflurane and LPS exposure. This was confirmed with a cytotoxic assay (determination of lactate dehydrogenase, LDH, Promega, Madison, WI, USA, data not shown).

Cell characteristics of mAEC

As seen in **Figure 1**, primary culture of mAEC represented both type I and II AEC, detected by real-time PCR (**Tab. 1**).

Co-exposure of AECII to LPS and sevoflurane: Effects on sodium transport via ENaC and Na^+/K^+-ATPase:

ENaC activity was assessed in AECII monolayer measuring ^{22}sodium (^{22}Na) influx. As shown in **Figure 2A**, stimulation with LPS impaired ^{22}Na-influx by 17.4% ± 13.3% SD ($p<0.05$) compared to control group. In the presence of sevoflurane, sodium influx into AECII improved in the LPS group, reaching control values ($p<0.05$).

Activity of Na^+/K^+-ATPase, measured by ^{86}rubidium (^{86}Rb) influx, revealed a 16.2% ± 13.1% ($p<0.01$) decrease of ^{86}Rb-influx upon LPS stimulation (**Figure 2B**). In LPS-stimulated AECII co-exposed to sevoflurane ^{86}Rb-influx reached values comparable to the control group ($p<0.01$).

Co-exposure of mAEC to LPS and sevoflurane: Effects on sodium transport via ENaC and Na^+/K^+-ATPase:

No difference in ^{22}Na-influx was observed in all four groups (**Figure 3A**).

mAEC showed an increased Na^+/K^+-ATPase activity of 23.7% ± 24.5% in the LPS group, 26.1% ± 38.6% in the sevo/LPS group (both $p<0.05$). Sevoflurane did not have a significant impact on LPS-injured mAEC (**Figure 3B**).

***In vivo* effect of co-stimulation with LPS and sevoflurane on ENaC and Na^+/K^+-ATPase mRNA expression**

mRNA of α-ENaC was decreased by 58% ± 26.9% in the propofol/LPS compared to propofol/PBS group ($p<0.05$) (**Figure 4A**). Sevoflurane co-conditioning did not impact on expression of α-ENaC mRNA. γ-ENaC mRNA was downregulated in both LPS groups compared to propofol/PBS: it decreased by 81.7% ± 12.9% ($p<0.01$) in the propofol/LPS and 71.7% ± 17.3% ($p<0.01$) in the sevoflurane/LPS group (**Figure 4B**) with no intergroup difference.

Despite an increased expression of α_1-Na^+/K^+-ATPase mRNA in LPS-treated compared to control animals (increase of 46.5% ± 114.6 in the propofol/LPS, and 99.4% ± 81.4 in the propofol/LPS group), values between all groups did not differ significantly (**Figure 4C**).

***In vivo* effect of co-stimulation with LPS and sevoflurane: Oxygenation index (pO_2/FiO_2)**

While LPS application impaired oxygenation in the propofol group, oxygenation could be maintained in sevoflurane/LPS-treated animals comparable to propofol/PBS (**Figure 5**): At 6 hours, propofol/LPS animals presented with an oxygenation index of 298 mmHg compared to 6 hours sevoflurane/LPS animals with 474 mmHg ($p<0.05$). At 8 hours the difference even increased with 198 mmHg in propofol/LPS animals to 454 mmHg in LPS animals with sevoflurane application ($p< 0.001$).

Wet/dry ratio of differently treated animals

A 27.7% ± 21.2% higher wet/dry ratio in animals treated with propofol/LPS compared to sevoflurane/LPS was observed ($p<0.05$) (**Figure 6A**). Sevo/LPS animals treated with amiloride showed similar wet/dry-ratios as the group without amiloride application (**Figure 6B**).

Discussion

With the current data two main results can be summarized: First, sevoflurane has a stimulating effect on the pump function of sodium channels in LPS-injured AECII *in vitro*. However, no such impact was observed in a mixed culture of type I and II AEC (mAEC), this cell composition rather reflecting an *in vivo* situation with predominantly type I cells in the lung. *In vivo* data underline these findings showing that the presence of sevoflurane does not influence edema resolution. Secondly, sevoflurane has a positive impact on the course of LPS-induced injury *in vivo*. Animals anesthetized with sevoflurane presented with a better oxygenation.

Transepithelial sodium transport plays an important role in fluid clearance in normal and injured alveoli. α-ENaC thereby seems to be crucial as α-ENaC-deficient mice died shortly after birth due to lung edema even without pulmonary inflammation [43]. In addition, previous *in vivo* experiments have demonstrated that blocking of epithelial sodium channels in a rat model of hyperoxia-induced lung injury significantly increased extravascular lung fluid volume [44]. In the situation of ALI alveolar fluid transport can be up- or downregulated [45]. Hypoxia inhibits transepithelial sodium transport in *ex vivo* lungs [16], while endotoxin A from Pseudomonas aeruginosa stimulates alveolar fluid clearance in rats [46], probably by cytokines-induced stimulation of sodium uptake. On the contrary, intratracheal application of endotoxin impaired alveolar fluid clearance in adult rats at 6 hours of injury [26,47]. Evidence from previous studies indicate that a complex network of inflammatory cytokines and chemokines mediate and modify the inflammatory process in lung injury including edema formation [48-50].

It is known that inflammation in AEC is mitigated by application of sevoflurane [25]. Our *in vitro* investigations in AECII showed that LPS-induced impairment of both, ENaC and Na+/K+-ATPase is reversed upon co-exposure to sevoflurane. These data suggest that active sodium transport and thus water transport can functionally be increased in injured AECII by administration of sevoflurane. So far only type II cells were considered as the important regulators for salt and water transport [51]. However, since both type I and II

cells AEC express sodium transport channels [52,53] also AECI might play an important role in water and salt homeostasis in the lung [52]. Therefore, after the positive findings in AECII, *in vitro* experiments regarding sodium transport were re-assessed in a mixture of type I and II cells, a setup, which more likely reflects the *in vivo* situation with only 5% of type II and 95% type I cells in the lungs. With this mixture of AEC (mAEC) no LPS-induced change or a significant influence of sevoflurane was observed for functionality of ENaC. For Na+/K+-ATPase we could show increased activity upon LPS exposure, while sevoflurane did not have any significant impact on its function. Therefore we conclude that AECI are not actively involved in water reabsorption with regard to sodium channels.

A previous study showed that oxygenation significantly improved using sevoflurane in a post-conditioning setup in a LPS-induced ALI model (intratracheally applied LPS, followed 2 hours later by application of sevoflurane compared to propofol anesthesia) [26]. The present promising *in vitro* results from AECII encouraged us to elucidate the question to what extent sevoflurane may influence either edema resolution or edema formation. We were able to show that wet/dry ratio in the sevoflurane-treated animals was significantly lower compared to the propofol/LPS group, linking better oxygenation to less alveolar edema. However, when blocking the activity of ENaC using amiloride, wet/dry ratio remained unchanged. This important result suggests that sevoflurane most likely does not improve alveolar fluid reabsorption, but rather acts on the inflammatory side inhibiting the production of inflammatory mediators, which in consequence decreases vascular permeability. If alveolar water absorption had been more important than edema formation one would have expected a clearly increased wet/dry-ratio in case of blocked ENaC [41].

Another interesting observation of the current *in vivo* experiments is the fact that co-conditioning with sevoflurane is more effective in amelioration of oxygenation than postconditioning with the volatile anesthetic [26]. This finding suggests that an early treatment with sevoflurane could inhibit the increase of permeability and attenuate injury-induced vascular leakage.

The present study has several limitations that need to be addressed. Discussion from *in vitro* experiments is limited as the interaction with cells of different character is missing. Another concern lies in the experimental setup of ALI used. Even if intratracheal application of LPS is defined as a relevant *in vitro* and *in vivo* animal model for lung injury, it does not fully represent ALI in patients. Therefore, conclusions cannot necessarily be translated to a clinical situation. Furthermore, due to the fact that lungs could not be utilized for both measurement of lung wet/dry ratios and lung RNA-analysis experiments had to be repeated using different animals. This of course may create a sample bias, which we tried to minimize by following our strict experimental protocols. Nevertheless, despite these limitations the present study provides new information regarding the protective effect of volatile anesthetics in ALI.

In conclusion, these data reveal that sevoflurane reverses the inhibitory effect of LPS on the function of ENaC and Na^+/K^+-ATPase in AECII *in vitro*. Sevoflurane exposure can positively influence the course of LPS-induced lung injury with regard to oxygenation. This effect, however, seems not to be mediated by increased fluid clearance, but rather by the anti-inflammatory properties of sevoflurane leading to less edema formation.

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Figure legend

Figure 1:

TaqMan real-time PCR of AECII and mAEC. Detection of type I and type II characteristic ion transporters. Values are mean ± standard deviation. N=6 per group.

Figure 2A:

^{22}Na-uptake via epithelial sodium channel ENaC, relative counts per minute in the different groups. AECII cells were incubated for 8 hours with or without lipopolysaccharide (LPS) and with or without sevoflurane (Sevo). Values are mean ± standard deviation. N=18 per group.

* $p<0.05$ vs other 3 groups

Figure 2B:

Na^+/K^+-ATPase activity measured by ^{86}rubidium uptake in alveolar epithelial type II cells (AECII), relative counts per minute. Cells were incubated for 8 hours with or without lipopolysaccharide (LPS) and with or without sevoflurane (Sevo). Values are mean ± standard deviation. N=18 per group.

** $p<0.01$ vs other 3 groups

Figure 3A:

^{22}Na-uptake via epithelial sodium channel ENaC, relative counts per minute in the different groups. A mixture of type I and II alveolar epithelial cells (mAEC) cells were incubated for 8 hours with or without lipopolysaccharids (LPS) and with or without sevoflurane (Sevo). Values are mean ± standard deviation. N=18 per group.

Figure 3B:

Na^+/K^+-ATPase activity measured by ^{86}rubidium uptake in mAEC, relative counts per minute. mAEC were incubated for 8 hours with or without lipopolysaccharide (LPS) and with or without sevoflurane (Sevo). Values are mean ± standard deviation. N=18 per group.

* $p<0.05$ vs control

Figure 4A:

Expression of the α-subunit of epithelial sodium channel (α-ENaC) mRNA in differently treated animals. Propofol/phosphate-buffered saline (PBS) served as control. LPS = lipopolysaccharide. Values are mean ± standard deviation. N=6 per group.

* $p<0.05$ vs other 2 groups

Figure 4B:

Expression of the γ-subunit of epithelial sodium channel (γ-ENaC) mRNA in differently treated animals. Propofol/phosphate-buffered saline (PBS) served as control. LPS = lipopolysaccharide. Values are mean ± standard deviation. N=6 per group.

** $p<0.01$ vs propofol/PBS

Figure 4C:

Expression of α_1-subunit of Na^+/K^+-ATPase mRNA in differently treated animals. Propofol/phosphate-buffered saline (PBS) served as control. LPS = lipopolysaccharide. Values are mean ± standard deviation. N=6 per group.

Figure 5:

Arterial oxygen partial pressure in mmHg of blood gas analysis in differently treated animals. Propofol/phosphate-buffered saline (PBS) served as control. LPS =

lipopolysaccharide. Blood gas measurements were taken at the time points 0, 2, 4, 6 and 8 hours.. Values are mean ± standard deviation. N=6 per group.

# $p<0.05$ vs time points 0, 2 and 4 hours

* $p<0.05$ vs time point 6 hours

Figure 6A:

Wet/dry ratio of rat lungs treated with lipopolysaccharide (LPS) and either sevoflurane or propofol for 8 hours. Values are mean ± standard deviation. N=6 per group.

* $p<0.05$

Figure 6B:

Wet/dry ratio of rat lungs. Animals were treated with lipopolysaccharide (LPS) and sevoflurane for 8 hours. Half of the animals received amiloride intratracheally in order to block ENaC. Values are mean ± standard deviation. N=6 per group.

Table 1:

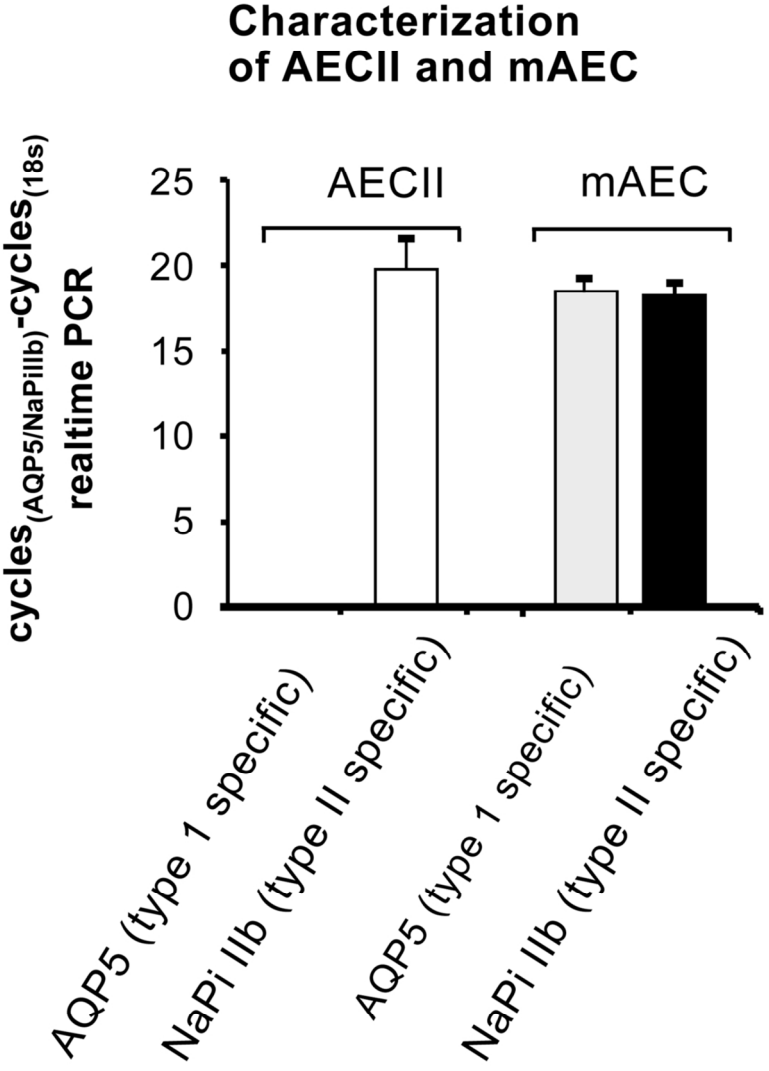
Primer sequences and probes used for TaqMan Real-time PCR

α-ENaC: alpha-subunit of the epithelial sodium channel, γ-ENaC: gamma-subunit of the epithelial sodium channel, α_1-Na^+/K^+-ATPase: sodium-potassium triphosphatasen (α_1-subunit), AQP5: Aquaporin 5, NaPi IIb: Sodium dependent phosphate cotransporter type IIb, 18S: housekeeping gene (ribosomal origin).

Tables

Table 1

Gene	Primer sequence and corresponding probe	Fragment size
α-ENaC	forward: 5' tgt gac tac cga aag cag agc 3'	102bp
	reverse: 5' agg ctt ccg aca ctt gga g 3'	
	probe #26	
γ-ENaC	forward: 5' agc aac acc cca act gga t 3'	93bp
	reverse: 5' agg att gct gca cac tga tt 3'	
	probe #26	
α_1-Na^+/K^+-ATPase	forward: 5' act tgg gca ctg aca tgg tt 3'	104bp
	reverse: 5' cac aag ttt gtc cgt ttt gg 3'	
	probe #26	
NaPi IIb	forward: 5' ccc agg aag agg agc aaa a 3'	72bp
	reverse: 5' tca gga gct ttg tgc caa c 3'	
	probe #64	
Aquaporin 5	forward: 5' gct ccg agc tgt ctt cta cg 3'	131bp
	reverse: 5' gcg ttg tgt tgt tgt tca gc 3'	
	probe #20	
18S	forward: 5' gga gag gga gcc tga gaa ac 3'	70bp
	reverse: 5' tcg gga gtg ggt aat ttg c 3'	
	probe #74	
probe #20	5' ctg gct gg 3'	
probe #26	5' cag ccc ag 3'	
probe #64	5' cca ggc tg 3'	
probe #74	5' ggc agc ag 3'	



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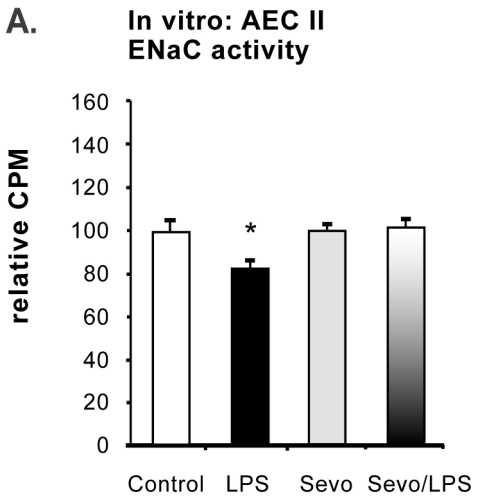

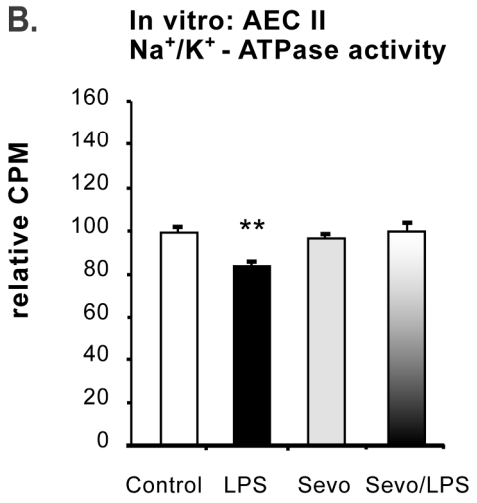

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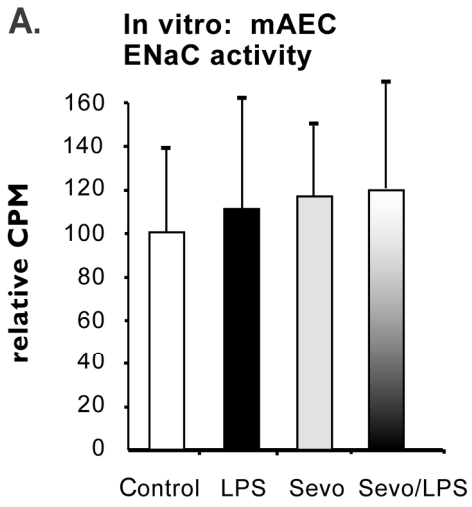

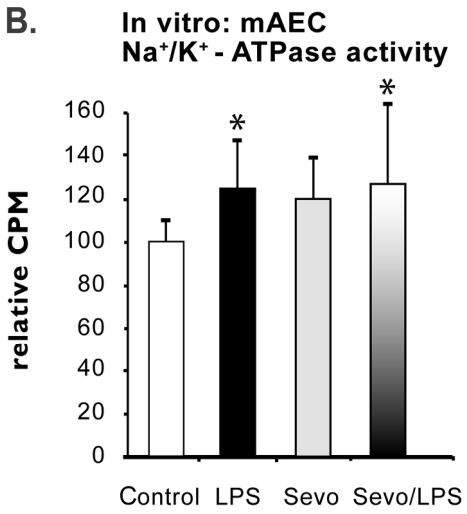

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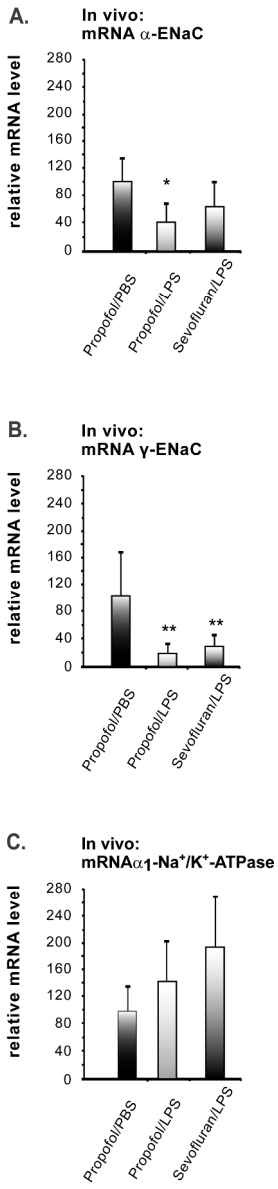

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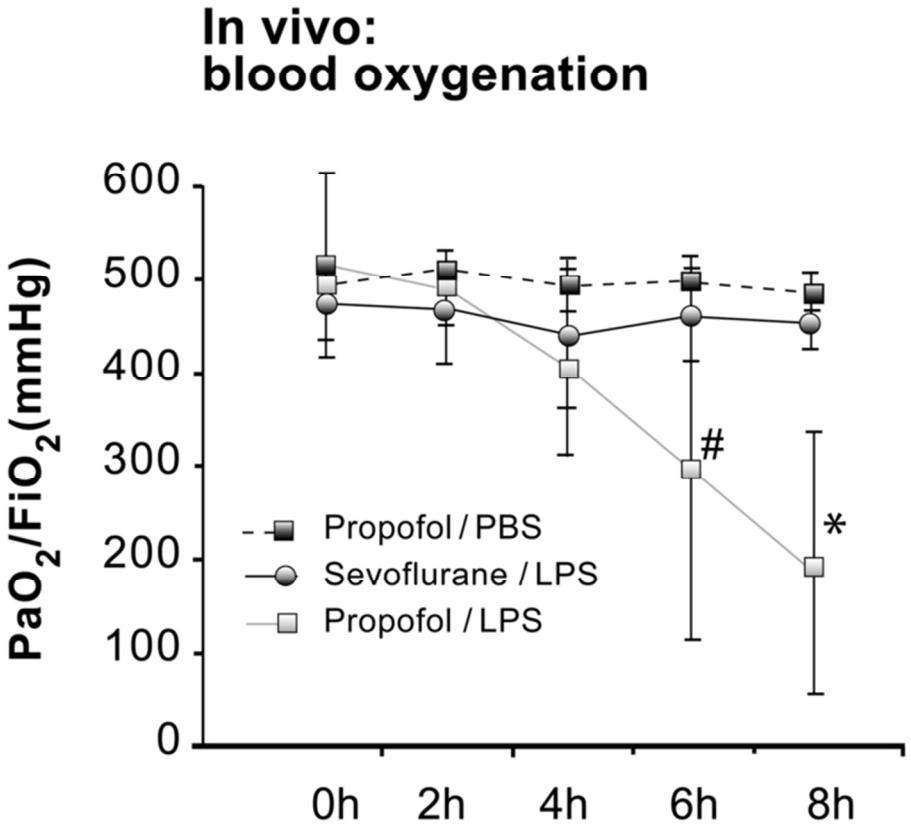

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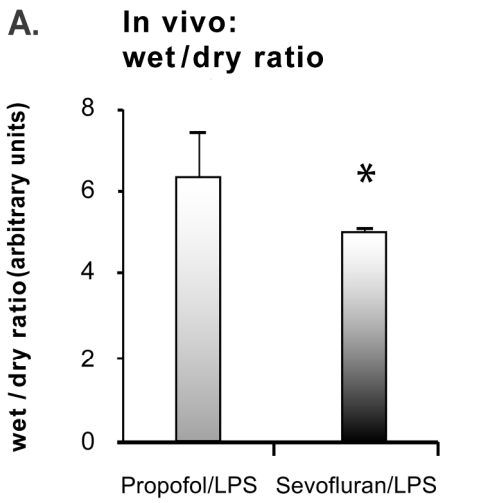

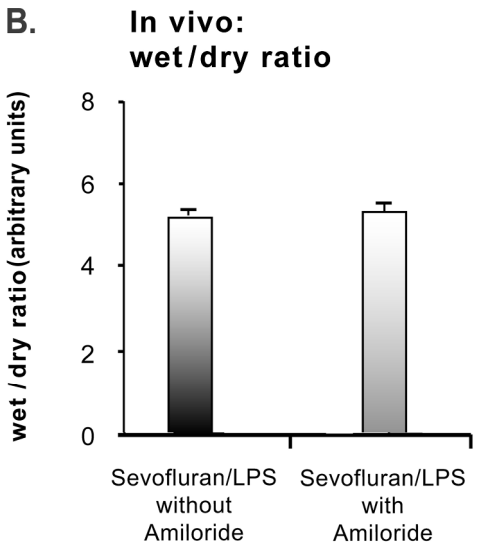

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